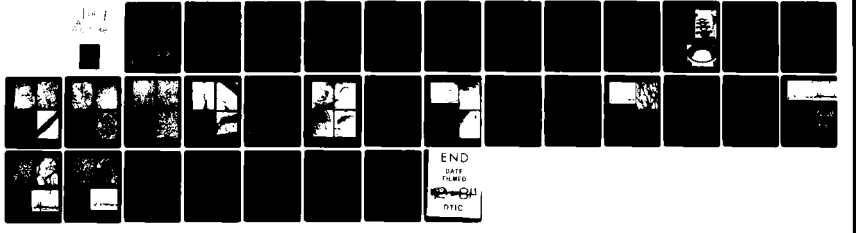
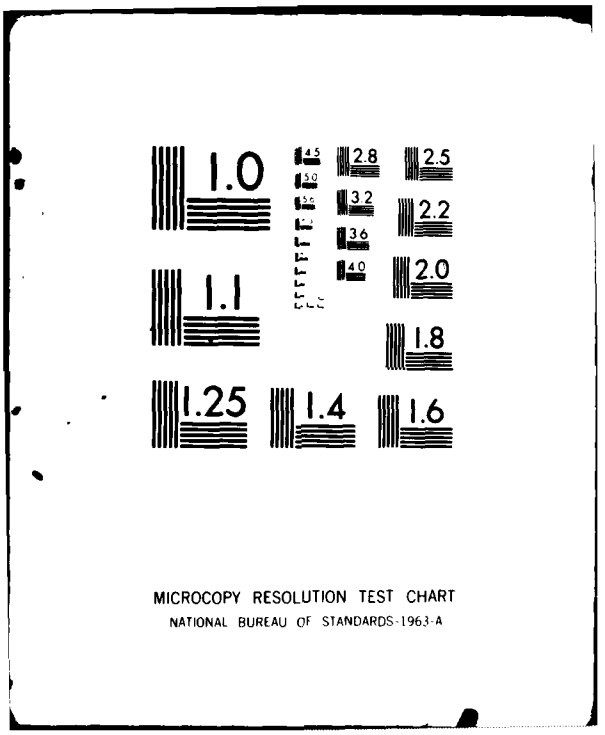
AD-A107 138 GRAMBLING STATE UNIV LA F/G 6/3
MONITORING CALCIUM IN TROUT EGGS EXPOSED TO HYDRAZINE.(U)
JUL 81 V HENDERSON AFOSR-81-0079
UNCLASSIFIED AFOSR-TR-81-0654 NL




1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

LEVEL II

1

Final Research Report

for

AFOSR GRANT NO. AFOSR-81-0079

entitled

Monitoring Calcium in Trout Eggs Exposed to Hydrazine

by

Vernon Henderson

Grambling State University, Grambling, Louisiana 71245

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER (18) AFOSR-TR- 81-0654	2. GOVT ACCESSION NO. AD-A107
	3. RECIPIENT'S CATALOG NUMBER 438
4. TITLE (and Subtitle) (6) Monitoring Calcium in Trout Eggs Exposed to Hydrazine	5. TYPE OF REPORT & PERIOD COVERED (9) Final Report 1 Feb - 1 Aug 1981
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) (10) Vernon Henderson	8. CONTRACT OR GRANT NUMBER(s) (15) AFOSR-81-0079
9. PERFORMING ORGANIZATION NAME AND ADDRESS Grambling State University Grambling, Louisiana 71245	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102F (16) 2312/D9 (17) D9
11. CONTROLLING OFFICE NAME AND ADDRESS (11) AFOSR Bolling AFB, D.C. 20332	12. REPORT DATE 1 July 1981 13. NUMBER OF PAGES 31
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Supported in part by Brookhaven National Laboratory, Biology Department, Upton Long Island, New York 11973, and Vernon Henderson.

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Trout Embryos
Trout Eggs
Hydrazine
Calcium

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This study was designed to determine if certain hydrazine-induced morphological abnormalities could be attributed to reduced levels of available calcium. Rainbow trout eggs (Salmo gairdneri) were exposed to 48 hours to 1.0, 2.0, 4.0, 6.0, and 8.0 mg/l of hydrazine. The responses to these concentrations were dose related. Eggs exposed to low concentration were less affected than those exposed to high concentration. Utilizing several staining techniques in combination with SEM, TEM and microelemental analysis, control muscle tissue

(Continued)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

391116

20. ABSTRACT--(Continued)

showed a higher calcium level than healthy muscle of the 8.0 mg/1 hydrazine treated group. Maldeveloped muscle of the 8.0 mg/1 group showed a higher level of calcium than healthy muscle of the same individual. The notochord of the 8.0 mg/1 group showed a higher calcium level than the control group. The chorion did not reveal a calcium level although structural modifications occurred in the treated groups which resulted in a softening.

Accession For	
NTIS GRA&I	X
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A	

SECURITY CLASSIFICATION OF THIS PAGE(When Data Entered)

ACKNOWLEDGEMENTS

I wish to thank the Air Force Office of Scientific Research for providing funds (AFOSR-81-0079) to support this study. I am indebted to Dr. Myron C. Ledbetter and Mrs. R. V. Wright, Biology Department, Brookhaven National Laboratory, Upton, Long Island, New York for their assistance and the use of their scanning electron microscope facilities. I am very grateful to Dr. George Schidlowsky and Mr. Richard Ruffin for the microelemental analysis and the preparation of specimen for same.

INTRODUCTION

The status of hydrazine as a mutagen, carcinogen, teratogen and toxicant is well documented in the literature (Greenhouse, 1976; Biancifiori et al, 1963; Toth, 1972; Hurly and Fisher, 1965; Fisher et al, 1978; Fisher et al, 1980; Klein and Jenkins, 1977 and Slonim, 1977). All classes of vertebrates were utilized in these studies. Recently, Henderson et al (1980, umpublished) observed by light microscopy that scoliosis, gaped mouth and downward-turned tail were induced in rainbow trout eggs exposed to hydrazine for 48 hours. Since hydrazine is capable of combining with calcium (Solnim, 1972), we speculated that these abnormalities and depressed muscular response to chorion pressure were related to the availability of calcium.

It is also known the hydrazine (Dost et al, 1979), in blood, is converted to N_2 very quickly during the first three hours after its introduction into the body of rats. Fifty percent of the hydrazine was converted to N_2 while in a cell-free hemoglobin system, the conversion was more than 80%. One basic question that can be raised about the toxic effects of hydrazine is, does it, per se, produce the morphological abnormalities or does its breakdown or converted products cause the changes. If an appreciable amount of N_2 is produced in the blood stream, one may expect a classic case of gas bubble disease (Marsh and Gorham, 1905) under such conditions. Some symptoms which characterize the disease include internal lesions, degenerative buccal epithelium and emphysema. Hemorrhages may occur in muscles, gonads, brain and nares. Data (Henderson et al, 1981) recently gathered indicated that hemorrhages occurred and hemoglobin synthesis was impaired in fathead minnow when treated with hydrazine. This suggested that N_2 was produced. Chapman (1980) reported that calcium

induces hemoglobin synthesis and that an imbalance ratio of exterior/internal calcium can alter the process. The above would suggest that even if some hydrazine is converted to N_2 and thereby produce the gas bubble disease, residual amounts could alter the ratio cellular ratio of exterior/internal calcium. It seems appropriate to examine organs or structural parts which require calcium in quantities that can be monitored with conventional instruments or techniques. The chorion, muscle and notochord tissues were the primary structural embryonic components used in monitoring calcium; however, selected physiological parameters and other structural entities were investigated to provide added evidence of calcium modulated functions.

METHODS AND MATERIALS

TEST ORGANISM

Twelve-day-old rainbow trout eggs (Salmo gairdneri) were obtained from Trout Lodge, Washington. Eggs were characterized by the presence of eye pigment, bile in the gut and a vent. The eggs were tempered in 10° C water prior to being grouped and subjected to various concentrations of hydrazine. A total of 6000 eggs were divided into six groups, with approximately 700 in the control and five experimentals.

WATER

Regular tap water was double filtered through Sears' Sediment (cellulose) and Taste-Odor (activated charcoal) filters. The filtered water was then used for the control and experimental groups. The pH ranged from 7.0 to 7.5, and the dissolved oxygen was between 7.2 and 8.0 mg/1. The $CaCO_3$ hardness was approximately 54.0 mg/1. The temperature ranged from 8-10° C in the egg chambers and 7-9° C in the Living Stream^R. The water for controls and experimentals were renewed at a rate of approximately 6 liters per hour.

RECIRCULATING WATER SYSTEM

a. Exposure Period

A continuous flow system in a 70-liter plastic containers constituted primary reservoirs for incubating the eggs. Six reservoirs were placed in a 570-liter capacity Living Stream^R (Frigids, Inc., Toledo, Ohio) to ensure a constant temperature. Each incubating chamber (Figures 1 and 2) consisted of a 26 cm diameter plastic funnel in which a one millimeter wire mesh platform was placed. A perforated aluminum disc provided support for the wire platform.

Each platform in the egg chamber was 15 cm in diameter. A hole was bored seven centimeters above the platform and a piece of plastic tubing inserted to provide an overflow outlet. This plastic tubing returned water to each reservoir. Each reservoir was provided with an overflow that conveyed water from the control and experimental chambers to the drain of the Living Stream^{R}. A Silent Giant Pump, placed in each reservoir was attached to a piece of plastic tubing which connected to the cone end of the funnel, providing force for circulating the water. A pinch clamp was attached to the plastic tubing between the pump and funnel in order to regulate the flow rate of approximately 440 ml/min. The eggs were exposed for 48 hours.

b. Purging Period

Embryos were transferred to filter tap water for 4 weeks. The incubating system was altered slightly. Six pieces of eight mm thick plexiglas measuring 27 cm by 58 cm with a 21 cm diameter hole cut in it, served to support the incubation chambers. Each chamber was provided with a pump for circulating the water within the Living Stream^{R}. The Living Stream^{R} was supplied with in-house double filtered water at 10 liters per hour. An overflow pipe placed in the drain of the Living Stream^{R} allowed water to flow continuously.

TOXICANT

Liquid anydrous hydrazine (H_2NNH_2) of 95+ purity was purchased from Eastman Chemical Company. The following concentrations of hydrazine were used in this study: 1.0, 2.0, 4.0, 6.0, and 8.0 mg/l. These quantities were weighed on a Mettler Analytical Balance, Model B. Water was added to each measured amount of hydrazine to bring the total volume to 70 liters. Seventy liters of each concentration were prepared every twelve hours and placed in 90-liter capacity plastic containers. The above concentrations of hydrazine were monitored colorimetrically using p-dimethylaminobenzaldehyde (DMBA) method

(Reynolds and Thomas, 1965). DMBA was prepared by mixing four grams with 100 ml of ethyl alcohol. Ten ml of concentrated HCL were added to the alcoholic mixture. Monitoring hydrazine was accomplished by taking nine milliliters of test water and mixing it with one milliliter of DMBA. This solution was allowed to stand for 20 minutes before determining the optical density at 460 μm using a Bausch and Lomb Colorimeter 20.

PREPARATION OF EMBRYOS FOR ELECTRON MICROSCOPY

All embryos were fixed initially in Karnovsky's (1965) following 24 hours of exposure to hydrazine, one, two and four weeks of purging. Embryos for regular SEM (Scanning Electron Microscopy) were treated with concanavalin A (100 μm/ml) for one hour in Tris buffer (pH 7.6). Embryos were treated with horse radish perioxidase (1 mg/ml) for 30 minutes and in DAB (Daiminobenzidene) for one hour. Next, embryos were treated with 2% osmium tetroxide buffered with .2 M sodium cacodylate with .1% calcium chloride and .2% sucrose. Embryos processed by OTO (Osmium-Thocarbizide-Osmium) method were exposed to thiocarbizide for 15 minutes, washed and treated with 1% osmium (Woods and Ledbetter, 1976). These embryos were infiltrated with Epon for three days and fractured while being cooled with liquid nitrogen. Embryos were dehydrated in a graded series of acetone, critical point dried in a Sorvall Critical Point Drying Apparatus and coated with gold (30 min-microamps). Embryos were observed with an AMR Scanning Electron Microscope, Model 1000A. Images were recorded on Kodak Commercial Film 2147, at an accelerating voltage of 10 kV, scanning rate 3 and spot size 8. Embryos for microelemental analysis were processed as the ones above through critical point drying. These embryos were coated with carbon and the analysis made using an AMR Scanning Electron Microscope, Model 1400, at an accelerating voltage of s0 kV, spot size 2. This microscope was equipped with a TN 20 TECO Northern X-Ray System utilizing a SILI Detector with

a 33 mm square window. Calcium curves and values were recorded on Polaroid film #665.

TEM (Transmission Electron Microscopy) preparations were postfixed in 2% osmium tetroxide containing 2.5% potassium pyroantimonate. Embryos were dehydrated in acetone, infiltrated with Epon-Araldite and embedded in same. Cured blocks were sectioned with a diamond knife. Thin sections (90 nm) were placed on uncoated, 200 mesh copper grids, double stained with uranyl acetate and lead citrate. Sections were viewed with a Philip 300, Electron Microscope at an accelerating voltage of 80 kV. Images were recorded on Kodak 4489 Electron Microscope Film.

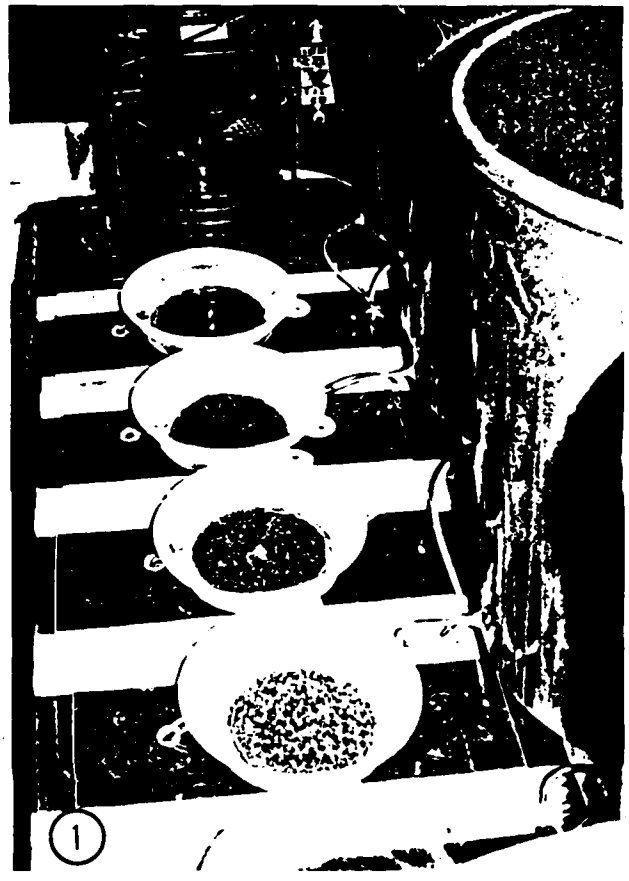


Figure 1. View of the Recirculating System for incubating eggs.

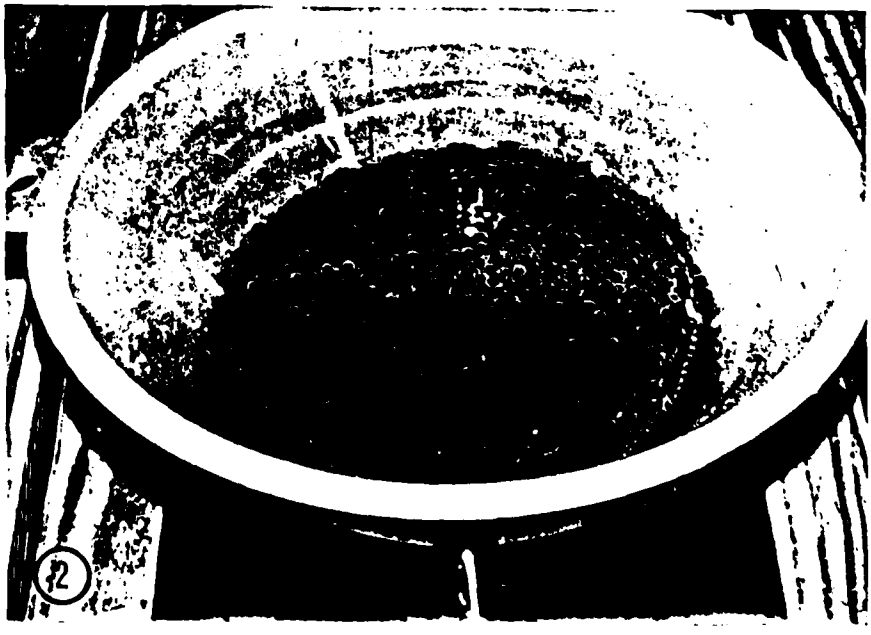


Figure 2. A single incubating chamber with eggs on the wire mesh platform.

RESULTS

CHORION

1. Control

An SEM examination of the exterior aspect of the chorion revealed a moderately textured surface (Figure 3). This surface had protein fibers that were slightly raised and cylindrical. The special staining technique did reveal the presence of glycoprotein or finely granular deposits. There was an absence of distinct pores, suggesting that there was a limited movement of materials across the chorion. The internal aspect (Figure 4) of the chorion was distinct from the exterior surface. A fibrous surface characterized this layer. The fibers were flat and interwoven among themselves. This structural feature gave the inner surface a dense meshwork appearance. There was a moderate amount of glycoprotein material on this surface. Some of this material was threadlike and others assumed a globular form. In some cases, the threads anastomosed with globular material. Fractured cross-sections (Figure 5) of the chorion showed fibers in parallel array. This character gave the chorion a spongy appearance.

When these chorions were removed as a part of the fixation procedure and for examining embryos in the live state, the chorion possessed good tensil strength. This feature was not quantitized. There was no apparent softening of the chorion. Microelemental analysis indicated an absence of calcium in the chorion. When stained for calcium using potassium pyroantimonate, there were not observable precipitate using light microscopy.

2. Treated

All embryos exposed to hydrazine had chorions that were structurally

altered. The external surface displayed raised cylindrical fibers in the 1.0, 2.0 (Figure 6) and the 4.0 (Figure 7) groups. Con A-HRP positive material was distributed uniformly over the surface. The fibers were closely packed. The fibers in the 6.0 and 8.0 mg/l (Figure 8) groups had collapsed with conspicuous pores scattered uniformly over the surface. The chorions of the 8.0 mg/l group displayed long cylindrical threads that appeared to be connected to the surface. Conspicuous amorphous deposits were observed in this group. The internal aspects of the chorion displayed the same general meshwork as control but the fibers were altered. The fibers were cylindrical and formed a more open or loose work (Figures 9, 10, and 11). The greatest amount of A-HRP positive material was observed in the 4.0 mg/l group, although it was observed in all treated groups. Cross-sectional views of chorions from the three groups: 4.0 (Figure 12), 6.0 (Figure 13), and 8.0 (Figure 14) mg/l revealed distinct changes in the patterns of the fibers. The 4.0 mg/l showed more anastomosing while the 8.0 mg/l showed more compactness and derrangement than any other group. The chorion fibers of the 8.0 mg/l group were oriented in the middle aspect so that they were perpendicular to the long axis of the inner and outer aspects of the chorion. The fibers with this different orientation were conspicuously compact. The chorion of the 6.0 mg/l group possessed fibers that were in a basic parallel arrangement; however, the layers were not loosely arranged.

All chorions in the treated groups underwent a softening process with an accompanying reduction in tensil strength. There was an absence of calcium when this structure was subjected to microelemental analysis and when it was stained with potassium pyro antimonate.

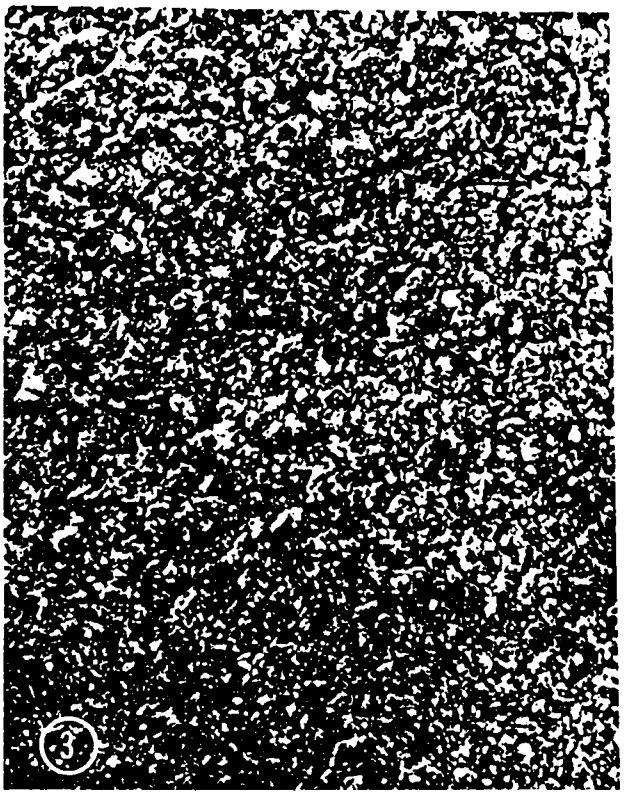


Figure 3. SEM micrograph of the external surface of chorion of control group (14 days old). Arrows indicate granular deposit of Con A-HRP material. X=2190.

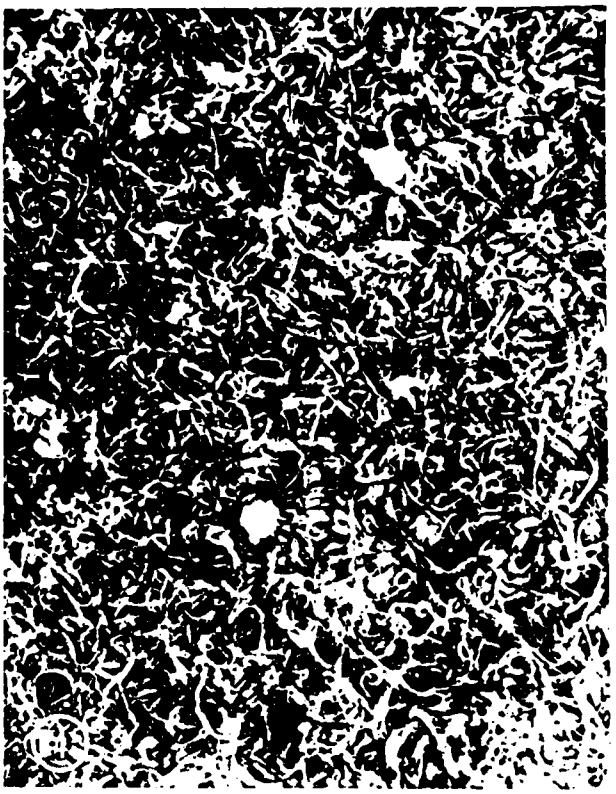


Figure 4. SEM micrograph of the internal surface of chorion of control group. X=2150.

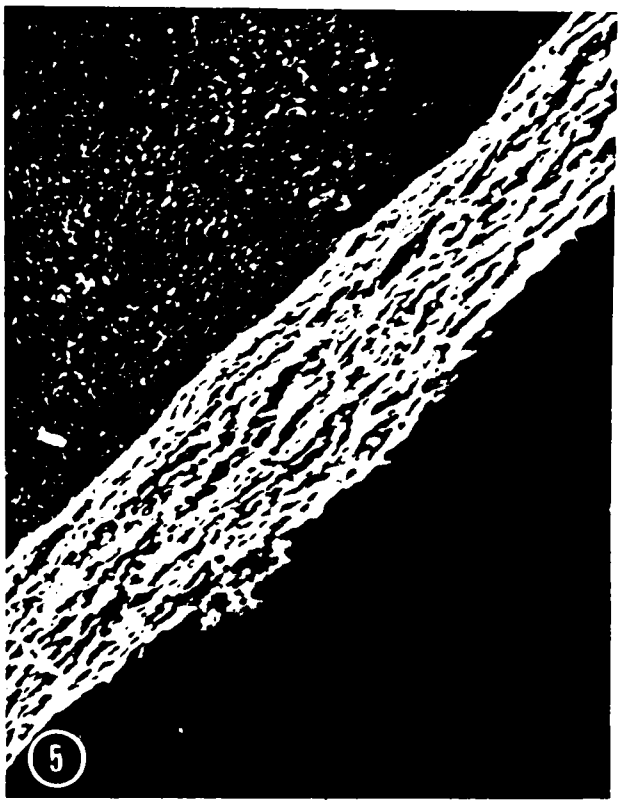


Figure 5. SEM of fractured surface of chorion (14 days old) of control group. Note the parallel arrangement of the fibers. X=100.

Figure 6. SEM micrograph of 2.0 mg/l group. The internal surface of chorion shows raised fibers. X=520.

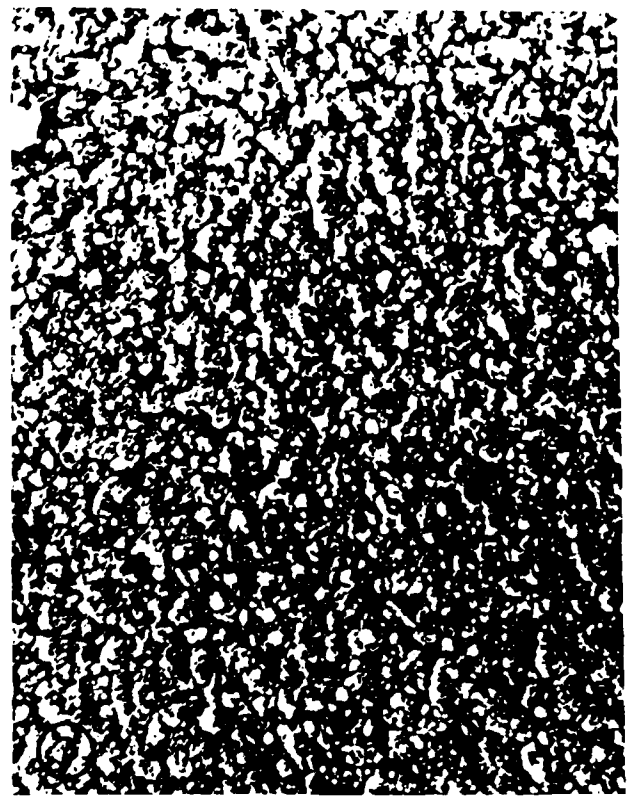


Figure 7. SEM micrograph of 4.0 mg/l group. X=550.

Figure 8. SEM micrograph showing collapsed chorion fibers of 8.0 mg/l group. X=5200.

Figure 9. SEM of internal chorion surface of 2.0 mg/l group. Note the loose meshwork formed by the chorion fibers. Embryos were 14 days old. X=5000.

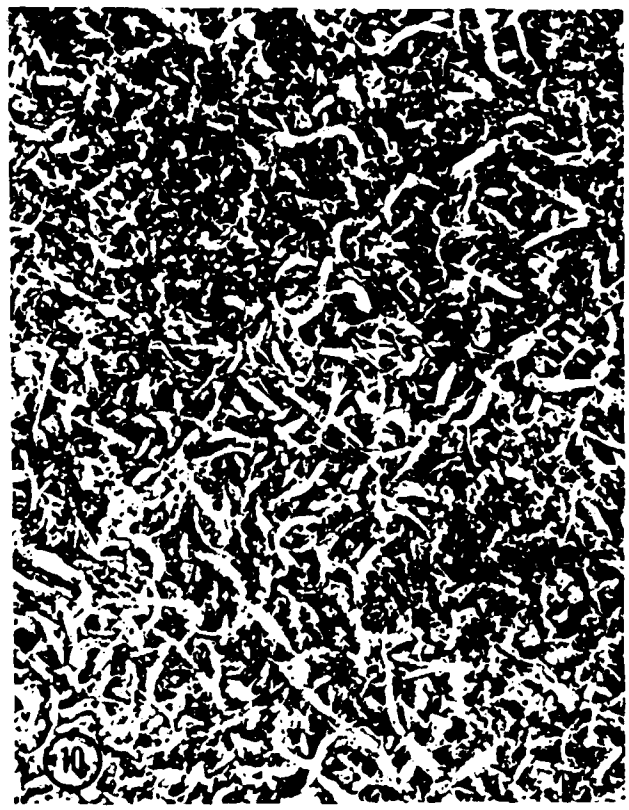


Figure 10. SEM of internal chorion surface of group treated with 4.0 mg/l. X=520.

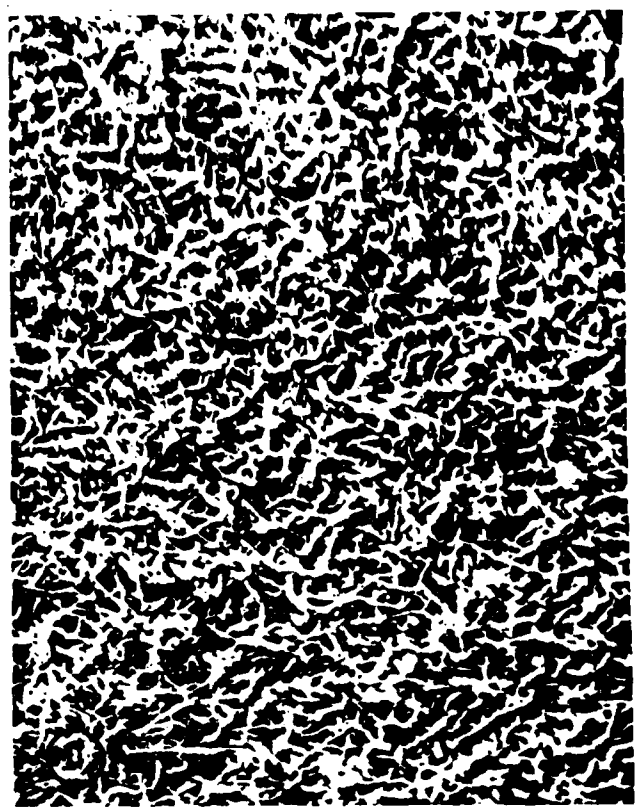


Figure 11. SEM of internal chorion surface of group treated with 8.0 mg/l. X=2100.

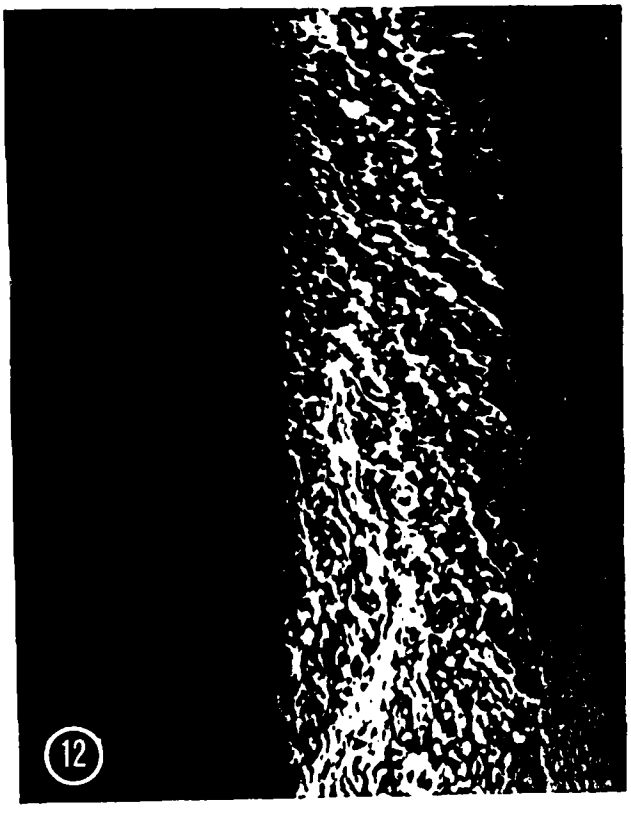


Figure 12. Cross section of fractured chorion of eggs treated with 4.0 mg/l of hydrazine. Fibers possess same parallel pattern as control but more compact. X=1100.

Figure 13. Cross section of fractured chorion of eggs treated with 6.0 mg/l hydrazine. Fibers of chorion are more compact than the control. X=1120.

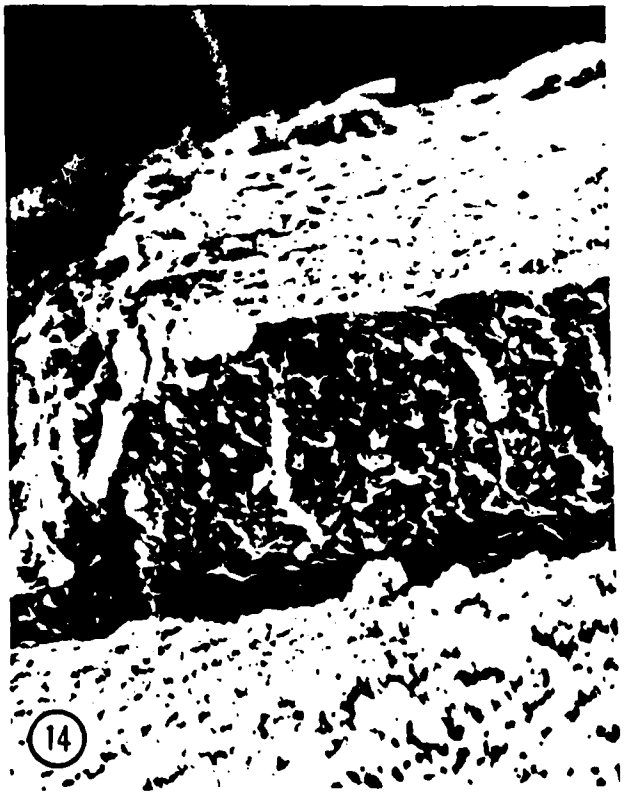


Figure 14. Cross section of fractured chorion of eggs treated with 8.0 mg/l hydrazine. Note the perpendicular arrangement of fibers constituting the middle portion of the chorion. X=2200.

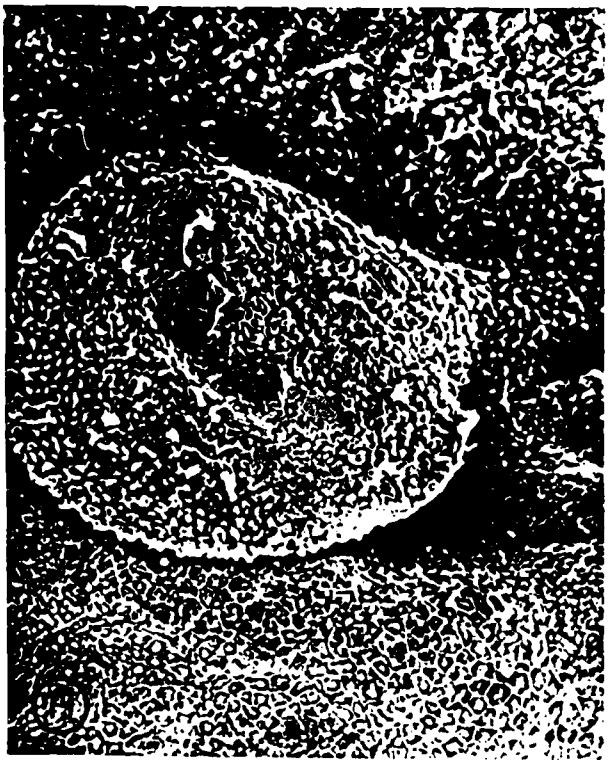
FIN SYSTEM

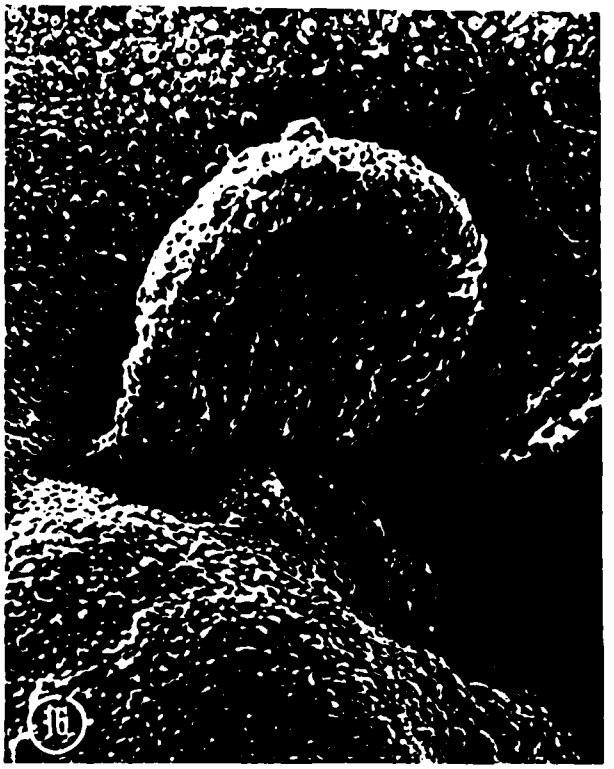
1. Control

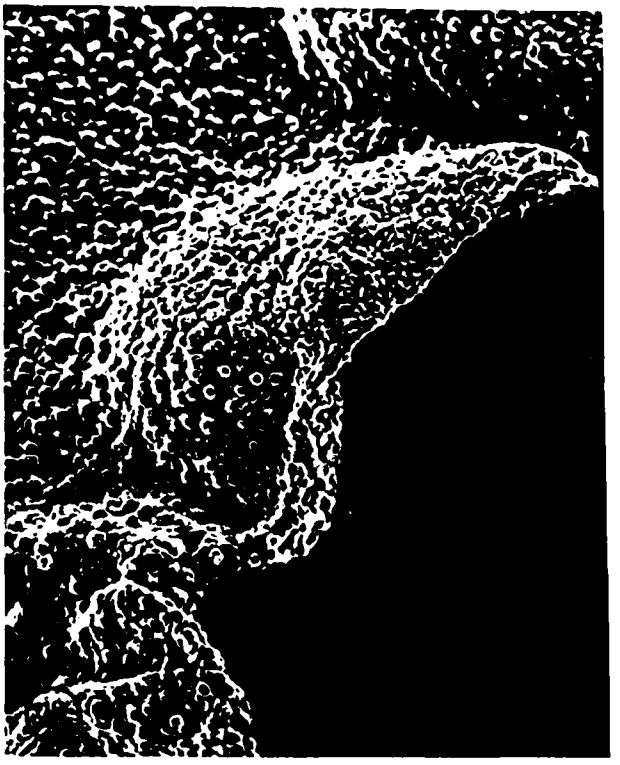
The pectoral, dorsal, and caudal fins developed the normal complement of connective tissue support. The pectoral fin (Figure 15) showed a surface with cells displaying an undulating edge. Light microscopy revealed supporting elements in 14-16 day old embryos, initially with a condensation of connective tissue. At the end of four weeks of purging, the pectoral fins were beating rhythmically with ray elements quite visible. The dorsal and caudal fins were derived from the fin fold. The latter was no longer present at the end of the purging period. There was an absence of any type of structural abnormalities in any of these fins.

2. Treated

The fin system in all hydrazine exposed embryos developed similar abnormalities. The pectoral fin showed an absence of the usual structural support. The fins curled at their periphery by the second week of purging and the condition persisted for the duration of the study. The edges simply folded inwardly or outwardly (Figure 16); cells piled-up, forming a thickened ridge. The dorsal and caudal developed minimally, only a slight indication of indentation suggested their presence while the fin fold still remained present. The caudal fin (Figure 17) never assumed its definite shape, the fin fold (Figure 18) thickened from the dorsal to the anal. These features or lack of normal structural components did not completely eliminate the movement of the pectoral fin; however, the amplitude of movement was greatly reduced. If the curl of the pectoral fin were outward, it appeared to be beating backwards. There was no apparent distinction among the other groups with regard to the structural modification of the pectoral, dorsal or caudal fins. There was no observable pectoral fin movement in the 2.0, 4.0, 6.0 or 8.0 mg/l groups.




16



JAW-OPERCULUM

1. Control

Fourteen-day-old embryos showed a normal perforated oral plate surrounded by the upper and lower jaws. The hyobranchial apparatus was well established. Development of these parts continue at a normal rate. At the end of four weeks of purging, the jaws were well developed (Figure 19), the opercular series were identifiable with joints well demarcated. Opercular movement was quite distinct. The mouth opened and closed, permitting water to pass off the gills.

2. Treated

These embryos, at 14 days, showed signs of depressed development. The upper and lower jaws were flat and were fused from near the original perforation to a distance half way to the angle of the jaw. The jaws did not move, the mouth was permanently gaped. This condition did not improve as development progressed. The gaped mouth (Figure 20) became more conspicuous during the four-week purging period. The upper jaw did not show a division of the premaxillary and supplemental maxillary. The operculum was without division; therefore, joints did not develop. A few raised areas (Figure 21) represented bone development or centers of ossification. The single bone or undivided operculum did not move. Embryos in the 1.0 mg/l group showed very little movement while the ones in the 2.0, 4.0 and 6.0 mg/l did not show any. The 8.0 mg/l embryos did not show movement throughout their survival period.

HATCHING AND SURVIVAL

1. Control

Embryos in this group began hatching on day 22 and by day 24, hatching was 90% complete. By day 26, 100% hatching had been accomplished. All of the hatched embryos were free of their chorion, there was no indication of partial hatching, that is, the chorion ruptured but part of the embryo was still inside

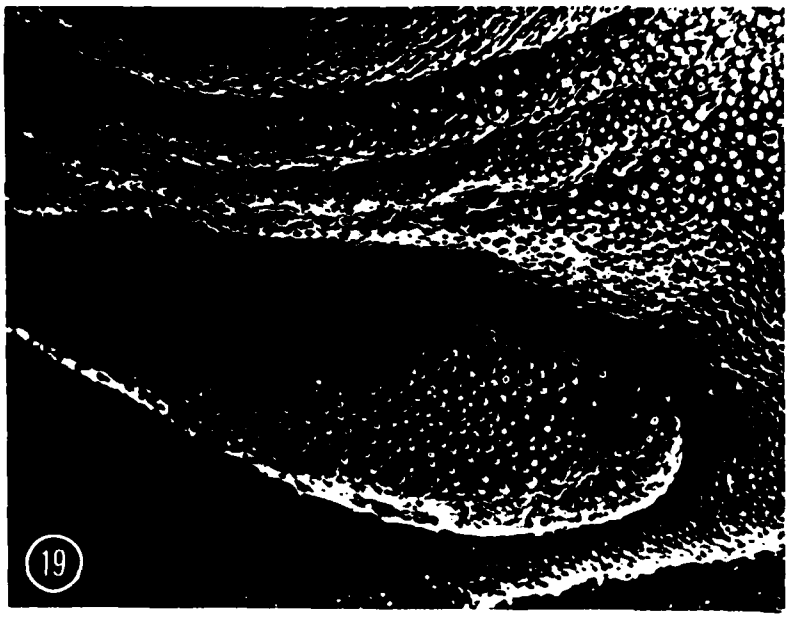


Figure 19. A control embryo (5 weeks old) with normal developed upper and lower jaws. X=116.

Figure 20. A 1.0 mg/l treated embryos (5 weeks old) with gaped mouth. X=62.

Figure 21. Surface view of undivided operculum, from 5-week-old embryo. X=62.

the chorion. These hatched embryos showed good body movement and responded well to being transferred via capillary tubing. Vigorous movement characterized this group for the duration of the experiment. Seventy-nine percent hatching success and 89.5% survival were recorded for this group.

2. Treated

The hydrazine exposed groups had hatching rates that were quite different from the control. The 1.0 mg/l was 20%, the 2.0, 4.0 and 6.0 mg/l were 10% on day 24, while the 8.0 mg/l was 0%. On day 26, the hatching rate was 75% for the 1.0 and 2.0 mg/l groups, 60% for the 4.0 mg/l group, 50% for the 6.0 mg/l group and 0% for the 8.0 mg/l group. Very little body movement was observed in the 1.0 mg/l group and no detectable movement was observed in the 2.0, 4.0 and 6.0 mg/l groups. All embryos in the 8.0 mg/l group were dead, some eggs had ruptured chorions but did not produce viable larvae. By day 28, embryos of all groups, except the 8.0 mg/l, had completed the hatching process. Table 1 compares the hatching rates of the treated group with the control. The differences are significant at the .001 level using the chi-square test. The survival rates (Table 1) were significant, only embryos of the control group were viable at the conclusions of four weeks of purging.

MUSCLE

1. Control

Embryos in this group displayed rhythmic contraction throughout this experiment. Response to pressure applied to the chorion was rapid and distinct. Hatched embryos or larvae showed body movements that resulted in good swimming behavior. The swimming was extremely vigorous when attempts were made to capture the embryos or when they were confined to capillary tubing. Examination of Con A-HRP-OTO processed embryos (hatched, 3-4 weeks of puring) revealed uniform muscle tissue. Myotubules were developing within the myoblast. There was an

TABLE 1

HATCHING AND SURVIVAL RATES OF EMBRYOS
BOTH UNTREATED AND TREATED

HYDRAZINE CONC mg/l	NO.	HATCHING SUCCESS* %	SURVIVAL RATE* 4 WEEKS POST-TREATMENT %
Control	787	79	89.5
1.0	1031	75	0
2.0	688	71	0
4.0	888	63	0
6.0	794	57	0
8.0	1324	0	0

*Significant at the .001 level using Chi-Square Test.

absence of heavy deposits of positive Con A-HRP material. Microelemental analysis showed a calcium peak (Figure 22) with particle counts of 2689/100 sec, recorded at 680 kev. TEM examination of similar tissue (Figure 23) for which a microelemental analysis was determined but stained with potassium pyroantimonate, revealed conspicuous deposits. These deposits, representing location of calcium, were distributed through the myoblast and within the sacroplastic reticulum.

2. Treated

Embryos of the 1.0 mg/l group displayed very little spontaneous muscular contractions, there was an absence of a response to chorion pressure and a sluggish response to touch of the body. All other groups showed no detectable muscular movement or response to chorion pressure or touch. Examination of muscle cross sections with SEM revealed deterioration ranging from very small in the 1.0 mg/l group to severe in the 6.0 mg/l. Con A-HRP technique revealed conspicious deposits within the myoblasts. There were always healthy appearing muscles in all groups, the greatest amount in the 1.0 mg/l group and the least

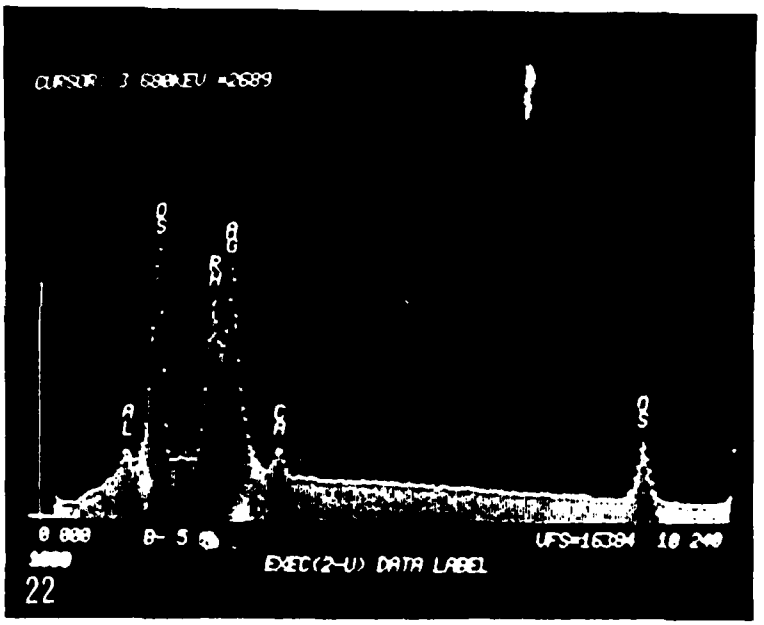


Figure 22. Kα X-ray line scan showing calcium peak in control muscle (embryo is 5 weeks old).

Figure 23. TEM micrograph of control muscle. Embryo was 5 weeks old. Arrows indicate sites where calcium is located.

in the 6.0 mg/l group. Microelemental analysis of the 6.0 mg/l group revealed high levels of calcium in severely deteriorating muscle (Figure 24) tissue while healthy muscle tissue (Figure 25) in the same specimen showed lower calcium levels. The particle count for the deteriorating muscle was 2649/1000 sec and 1508/1000 sec for the healthy muscle. TEM examination of similar tissue areas in the 6.0 mg/l group revealed necrotic tissue and a presence of conspicuous deposits of calcium pyorantimonate (Figure 26), supported the findings of the microelemental analysis.

NOTOCHORD

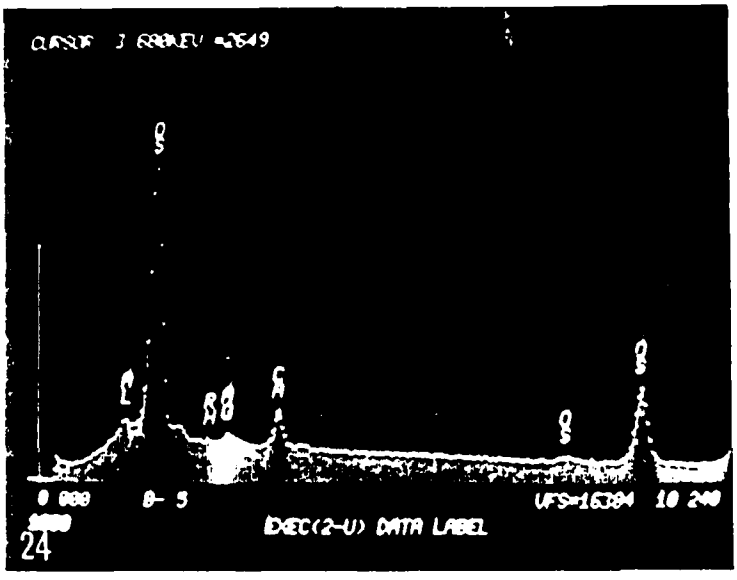
1. Control

The notochord of control embryos, processed with Con A-HRP, displayed cells with few large fibers and globular deposits at day 16 (Figure 27). The cells possessed a meshwork of fine fibers that were loosely arranged. In older embryos, the meshwork did not exist. A few fibers could be observed (Figure 28) spanning the diameter of the notochordal cells. A few collapsed membrane, along with amorphous deposits constituted the structural material of these cells. Microelemental analysis showed a calcium peak (Figure 29) with a particle count of 830/1000 sec.

2. Treated

All treated embryos showed structural modifications when compared to the control. The structural features rendered by the hydrazine exposure were a thickening of the meshwork and a compacting of the meshwork in 16-day-old embryos (Figure 30). There appeared to be a large number of globular deposits, a few in the 1.0 mg/l group and a large number in the 8.0 mg/l group. In older embryos, after 4 weeks of purging, the notochordal cells no longer displayed the meshwork but rather displayed fibers that were heavily coated with a Con A-HRP positive material. The largest number (Figure 31) of fibers appeared in the

6.0 mg/l group. The 2.0 and 4.0 mg/l groups displayed an intermediate number of fibers. Microelemental analysis of the 6.0 mg/l embryos revealed a calcium peak (Figure 32) with a 2602/1000 sec particle count. All treated embryos were scolitoic. All embryos showed positive Con A-HRP reaction in the notochord.



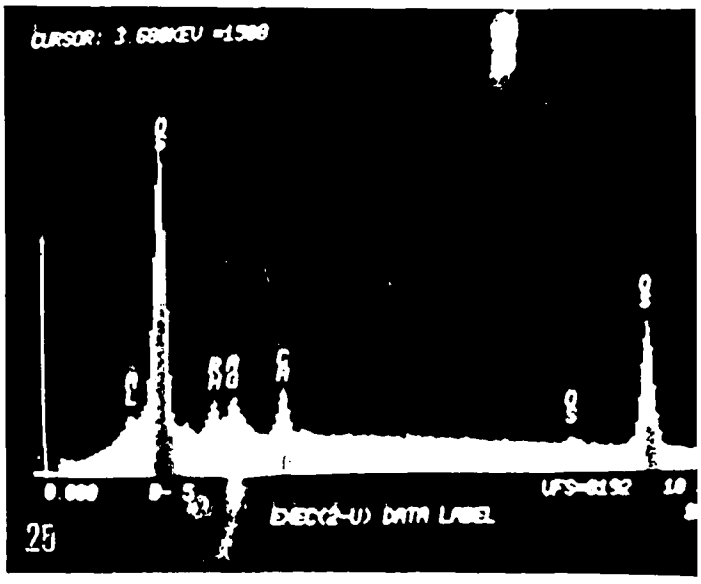
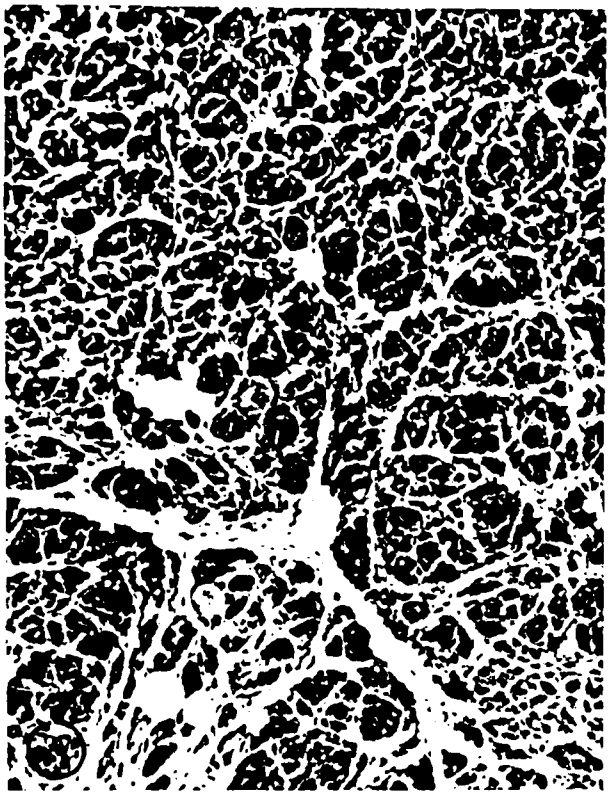


Figure 24. Kα X-ray line scan showing calcium peak in deteriorating muscle of treated embryo (5 weeks old), 6.0 mg/l group.

Figure 25. Kα X-ray line scan showing calcium peak in a healthy muscle of treated embryo (same embryo as in Figure 24), 6.0 mg/l group.

Figure 26. TEM micrograph of treated embryo (5 weeks old), 6.0 mg/l group. Fine precipitate represents calcium sites. Note the dark staining of necrotic muscle myofibrils.



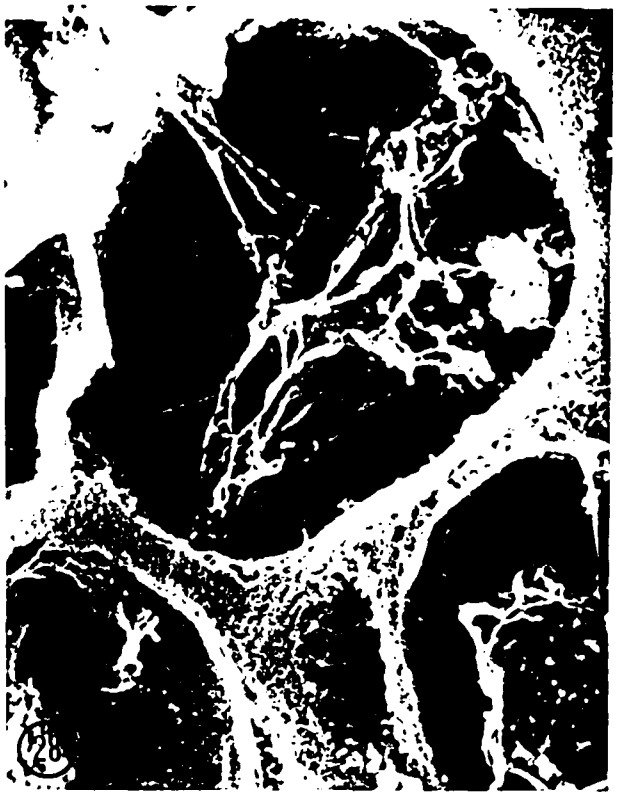


Figure 27. SEM showing notochordal cells at day 16 from control embryos. X=820.

Figure 28. SEM showing notochordal cells in hatched embryo (5 weeks old) from control group. Note the scarcity of fibers. X=235.

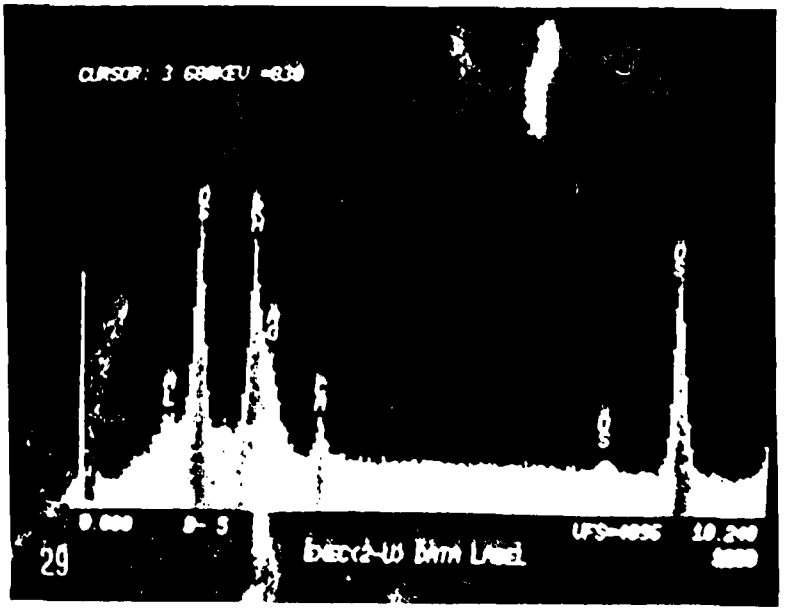


Figure 29. Kα X-ray line scan showing calcium peak of notochordal cells from control group.

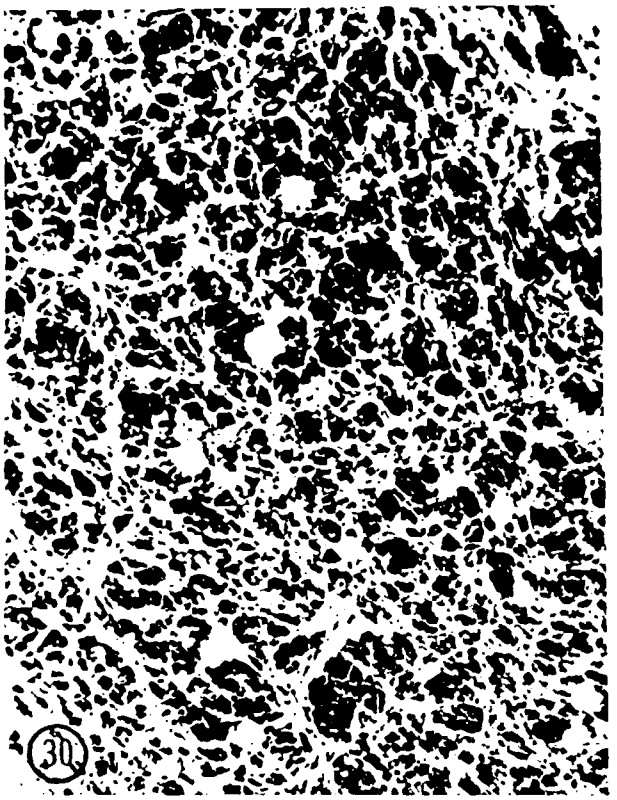


Figure 30. SEM showing notochordal cells at day 16 from 8.0 mg/l group. X=5200.

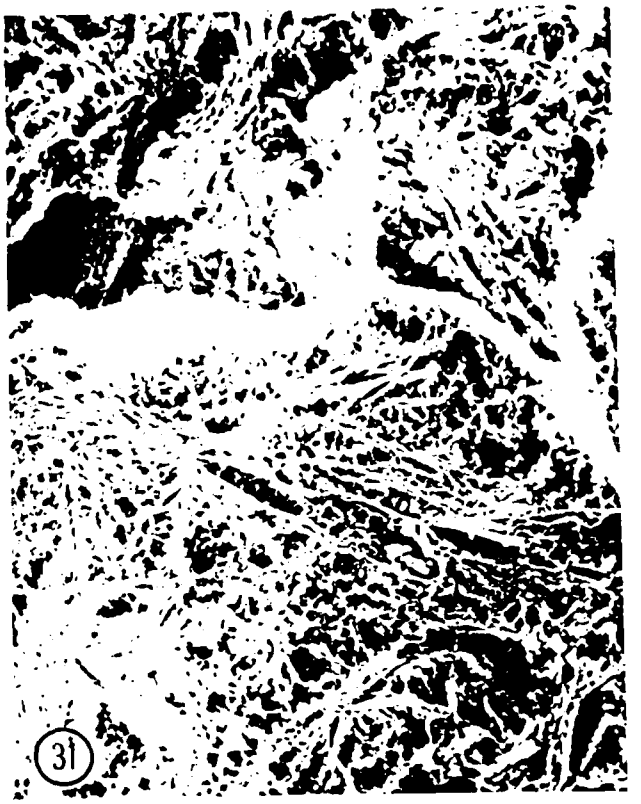


Figure 31. SEM showing notochordal cells in hatched embryo (5 weeks old) from 6.0 mg/l group. X=2300.

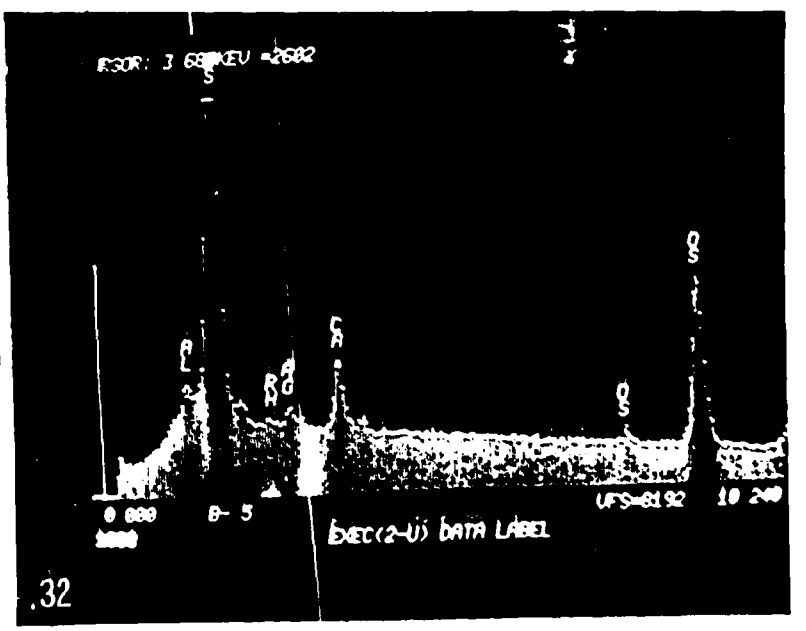


Figure 32. Kα X-ray line scan showing calcium peak of notochordal cells from 6.0 mg/l group.

DISCUSSION

Certain toxic effects of hydrazine are produced when it is converted into nitrogen. Gaseous nitrogen increases the total gas pressure which causes the classic bubble disease (Weitkemp and Katz, 1981). The primary symptoms are emphysema and hemostasis. The latter produces death due to anoxia. Emphysema and hemostasis were observed in this study but were not included as a part of this report. Since gas bubble disease is not a direct result of an interference in calcium metabolism, its role in producing abnormalities and death was not investigated. However, the gas bubble disease needs to be investigated to characterize the specific structural and physiological alterations associated with it.

Hydrazine's primary role in producing toxicity in rainbow trout eggs occurs when it combines with calcium (Slonim, 1977), thereby acting as a competitive anatagonist for divalent cations. Based on this kind of evidence, calcium was monitored in embryos exposed to various concentrations of hydrazine. All structures which require calcium either in a regulatory or structural capacity can be used to monitor calcium. If body functions or structures are altered after an exposure to hydrazine, one can infer that calcium levels are low. The chorion typifies this notion. The chorion underwent a softening process in all treated eggs. The fibers of the chorion reoriented and became tightly packed. Potts and Fleming (1970) noted that calcium elevations produced distinct structural changes in gill membranes. This appears to be the case with the chorion of hydrazine treated eggs. Calcium in this structure assumes a regulator role rather than being a structural component of the chorion. A reduction in available calcium can cause chorion fragility (Holcombe and Andrew, 1978);

Holcombe et al, 1979). The result of this study indicated a deposit of Con A-HRP material on the chorion but microelemental analysis failed to show calcium in either the control or treated. Perhaps the instrument did not have the capability to detect calcium at such low levels.

This study provides evidence to support the view that hydrazine interfers with rainbow trout embryogenesis by combining with calcium, making it less available. Normal muscle and bone development require calcium. The former requires calcium not only for structural needs but also for biochemical contraction. The controls presented a typical ultrastructural view of skeletal muscle while muscles of treated embryos were in various stages of deterioration. A combination of SEM, TEM and microelemental analytical techniques provided substantial evidence that the lack of calcium does not promote muscle development. It is known that decreased calcium inhibits myotubal formation (MacBride and Pryzbylski, 1980). One point should be noted, microelemental analysis and TEM, using potassium pyroantimonate provided the best monitoring scheme. The microelemental analytic procedure provides the best possible means for quantitizing data. Although employment (Dipolo et al, 1976) of metallochromic dyes have been used in conjunction with spectrophotometric analysis, this procedure does not allow one to obtain data showing calcium levels for healthy and deteriorating muscle. Unquestionably, TEM procedure provides the best qualitative information.

Bone structures are severely inhibited by a lack of calcium. Fin ray elements do not become elaborated, the mouth becomes strongly gaped, and the body becomes scoliotic. Data of this study suggest that notochordal cells develop high levels of calcium. It has been suggested that too much or too little calcium can alter certain differentiation processes (Chapman, 1980). The high level of calcium in the notochord can be attributed to poor circulation which is characteristic of this structure. Undoubtedly, the hydrazine, in treated

embryos, accumulated in the notochord with its high calcium load, released it and it remained there. Thickened fibers of the notochord would tend to retard an outward calcium flow. This would appear to be the reason for the high calcium level. Since bone growth is abnormal and the distribution of muscle forces is altered, scoliosis would be expected in a large number of treated embryos. One way to check this would be to use native rainbow trout where the incidence of scoliosis is higher than in some of the hybridized species. The gaped mouth was not analysed using fractured surfaces for examination by SEM and microelemental analysis. It would appear that this abnormality can be attributed to a lack of calcium which is required for normal muscle and jaw development. The opercular series of bone would fall into this same category.

The combination of SEM, TEM and microelemental analysis provides the best scheme for monitoring calcium. The SEM utilizing Con A-HRP is the best way to observe the matrix of notochordal cells. TEM can be utilized to view these cells but the number of specimen which could be examined would be fewer. TEM, using potassium pyroantimonate and microelemental analysis, must be utilized for direct qualitative evidence of calcium levels. Microelemental analysis is a rather involved procedure, but when used prudently with TEM, calcium levels can be properly assessed in healthy and unhealthy or deteriorating structures.

While the present study provides a means for monitoring calcium, more data must be collected. The use of chelating agents such as EGTA must be included at several different points, beginning with the exposure and ending with staining in an effort to incriminate calcium.

The total gas pressure must be monitored during egg treatment and incubation in order to determine how much of the hydrazine toxicity can be classified as primary as well as that which is a secondary manifestation.

REFERENCES

Biancifiori, C., Buciarelli, D., F. Santilli. 1963. Lung carcinogenesis for hydrazine sulfate in BALB/c female mice. Lav. Inst. Anat. Istol. Univ. Studi, Perugia: 115-128.

Chapman, L. 1980. Effects of calcium on differentiation of Friend leukemia cells. Develop. Biology, 79:243-246.

Dipolo, R., J. Requena, F. Brinley, I. Mullins, A. Carpa and T. Tiffert. 1976. Ionized calcium concentrations in squid axons. J. Gen. Physiology, 67:433-467.

Dost, F., D. Springer, B. Krivak. 1979. Metabolism of hydrazine. AMRL-TR 43, Wright-Patterson Air Force Base, Ohio.

Fisher, J., C. Harrah, W. Berry. 1980. Hydrazine: Acute toxicity to bluegills and sublethal effects on dorsal light responses and aggression. Trans. Amer. Fish, 109:304-309.

Fisher, J., C. Harrah, L. Weaver, W. Wingo. 1978. Acute and behavioral effects of hydrazine of Lepomis machorchirus. AFAMRL-TR-58, Wright-Patterson Air Force Base, Ohio.

Greenhouse, G. 1976. Evaluation of the teratogenic effects of hydrazine, methylhydrazine dimethylhydrazine on embryos of Xenopus laevis, the South African clawed toad. Teratology. 13:167-177.

Henderson, V., J. Fisher, R. D'Allessandris. 1980. Effects of hydrazine on the morphology and physical development in rainbow trout eggs. (Unpublished.)

Henderson, V., J. Fisher and R. D'Allessandris. 1981. Toxic and teratogenic effects of hydrazine on fathead minnow (Pimephales promelas) embryos. Bulletin Env. Contam. and Toxicology. (Accepted for Publication.)

Holcombe, E. and R. Andrew. 1978. The acute toxicity of zinc to rainbow and brook trout: Comparisons in hard and soft water. W. S. EPA Ecological Research Series. EPA60013-78094.

Holcombe, G., D. Benoit and E. Leonard. 1971. Long-term effects of zinc exposures on brook trout (Salvelinus fontialis). J. Fish Board Canada. 33:1731-1741.

Hurley, D. and K. Fisher. 1965. The structure and development of the external membranes in young eggs of the brook trout (Salvelinus fontialis). Canada. J. Zool. 44:173-190.

Karnovsky, M. 1965. A formaldehyde-glytaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biology. 27:137A.

Klein, S. and D. Jenkins. 1977. Environmental quality research: Fish aufwuchs bioassay. Second Annual Report. AMRL-TR-77, Wright-Patterson Air Force Base, Ohio.

MacBride, R. and R. Przbyhzski. 1980. Purified lectin from skeletal muscle inhibits myotubla formation. In Vitro. J. Cell Biology. 85:617-625.

Marsh, M. and F. Gorham. 1905. The gas diseases in fishes. Report of the US Bureau of Fish. 343-376.

Potts, W. and W. R. Fleming. 1970. The effects of prolactin and divalent ions on the permeability of water of Fundulus kansae. J. Exptl Biology. 53:317-327.

Reynolds, B. and A. Thomas. 1964. A colorimetric method for the determination of hydrazine and monomethylhydrazine in blood. Am. Ind. Hyg. Assoc. 26:527-531.

Slonim, A. 1977. Acute toxicity of selected hydrazines to the common guppy. Water Research. 11:889-895.

Slonim, A. 1972. Behavior of hydrazine compounds in hard and soft water. AMRL-TR-75. Wright-Patterson Air Force Base, Ohio.

Toth, B. 1972. Hydrazine, methylhydrazine and methylhydrazine sulfate carcinogenesis in Swill mice - failure of ammonium hydroxide to interfere in the development of tumors. *Int. J. Cancer.* 9:109-118.

Weitkamp, D. and M. Katz. 1980. A review of dissolved gas supersaturation literature. Trans. Amer. Fish Soc. 109:659-702.

Woods, P. and M. Ledbetter. 1976. Cell organelles at uncoated cyrofractured surfaces as viewed with the scanning electron microscope. J. Cell Sci. 21:47-58.


ILMED
8/1